SUPPORTING ONLINE MATERIAL

Materials and Methods

Animals and tissue collection: Animal care was in accordance with Duke University Medical Center institutional guidelines. Eyes were enucleated and quickly immersed in cold 2% paraformaldehyde (PFA) for 1 h. A slit was made in the enucleated eye by cutting around the ora serrata and fixation was continued in fresh 2% PFA at 4°C for another 1 h. The fixed eyecups were immersed in 30% sucrose (w/v) overnight at 4°C. The eyecups were embedded in OCT and cut on a cryostat to obtain 10 μm thick sections that were collected on slides coated with Vectabond (Vector labs). In in vivo knockdown experiments, eyecups from control and shRNA injected mice were embedded side by side in OCT, cut and collected on the same slide to minimize variations in quantification of immunofluorescence intensities. For observation of the olfactory epithelia, heads of neonatal mouse pups were first fixed in 2% PFA at 4°C for 3h, immersed in 30% sucrose, embedded in OCT and cut to obtain 15 μm thick coronal sections. Mouse sperm was isolated from the epididymis and vas deferens of adult males that had been housed separately from females. Transgenic *Xenopus* tadpoles at development stages 43-54 were fixed in 4% PFA, immersed in 30% sucrose, embedded in OCT and cut on a cryostat to obtain 12 μm thick sections for immunofluorescence analysis.

cDNA: cDNA encoding human CNG-β1(S*1*) was isolated by PCR from a retinal cDNA library (Clontech) and introduced into the XhoI and Not I sites of pEGFPN1 (Clontech). The sequence encoding the 28 C-terminal amino acids was deleted using PCR. Neurofascin fusion proteins were constructed by introducing PCR fragments coding for the cytoplasmic CNG-β1 N-terminus (amino acids 1-654), C-terminus (amino acid 1041-

1251) or mutated C-terminus using ApaI and Not I sites in a HA-neurofascin plasmid described in an earlier study (S*2*). Deletion mutations were obtained by PCR. Alanine mutations were introduced using the Quickchange mutagenesis kit (Stratagene). PCR was used to introduce either the 14 amino acids encoding the ankyrin binding site of human β-dystroglycan (DAG) (see Fig 3 C)) or the DAG ankyrin binding site with the mutation IIF798AAA which disrupts binding to ankyrin-G (S*3*), were introduced downstream of P1217 of the CNG-β1 polypeptide. All mutations were confirmed by sequencing. For transgenic expression in *Xenopus*, the wild type and mutant CNG-β1 cDNA were subcloned behind the 5.5 kb opsin promoter in a vector containing a γ-crystallin-GFP cassette to facilitate identification of transgenic animals (S*4*). Mouse ankyrin-G shRNA pFIV plasmid and control pFIV plasmid have been described before (S*3*). A cDNA encoding bovine CNG-α1 in pcDNA3 was a gift of Dr. Robert Molday (University of British Columbia, Canada).

Retinal electroporation: Electroporation of newborn CD-1 mouse pups was performed as described (S*5*). Briefly, mice were anesthetized by chilling on ice and a small incision was made in the sclera near the lens using a 30-gauge needle. Plasmid either encoding ankyrin-G shRNA or control pFIV (3μg/μl; (S*3*)) was mixed with pCAGGS-GFP (0.3μg/μl; (S*5*)) in PBS containing 0.1% fast green as a tracer. Then, under a dissecting microscope, 0.5 μl of the DNA mixture was injected into the subretinal space using a 33-gauge blunt-ended needle fitted to a Nanofil syringe (WPI, Inc) through the previously made incision. After DNA injection, tweezer-type electrodes (BTX, model 520, 7 mm diameter) smeared with a thin coating of a conductive gel (Signa Gel, Parker Laboratories) was placed gently on either side of the heads of injected pups, and five 80V

square pulses of 50 ms duration with 950 ms intervals were applied using a BTX ECM 830 pulse generator (Harvard Apparatus).

Antibodies: The rabbit polyclonal antibodies against *Aqueoria Victoria* green fluorescence protein (GFP), ankyrin-G, and ankyrin-B have been described before (S*6-8*). Monoclonal NKA pan alpha subunit antibody was from Affinity Bioreagents. Hybridoma supernatants containing monoclonal antibody against CNG-β1 (Garp 8G8; (S*9*)), CNG-α1 (Pmc1D1; (S*10*)) and rhodopsin (1D4; (S*11*)) were a generous gift from Robert Molday (University of British Columbia, Canada). Goat polyclonal CNG-α1 antibody (C-20) was from Santa Cruz Biotechnology, Inc. Rabbit polyclonal antibody against the HA epitope was from Covance.

Immunofluorescence: Immunofluorescence analysis of eye sections was performed as described before (S*12*). Briefly, mouse retina sections were permeabilized using 0.1% triton X-100, with 5% normal goat serum, and incubated with the appropriate primary and secondary antibodies. Immunofluorescence of HEK 293 cells was performed as described (S*2*). In recruitment assays, the cells were immunostained using the rabbit anti-GFP to detect ankyrin-G-GFP or ankyrin-B-GFP, the monoclonal Garp8G8 to detect CNG-β1 and a goat polyclonal antibody to detect CNG-α1. Images were collected using a 100 X objective (N.A. 1.45). To detect CNG-β1 constructs in *Xenopus* retina, tissue sections were permeabilized with 0.5% Triton X-100, blocked with 5% normal goat serum, incubated with a mouse monoclonal anti-CNG-β1 antibody (Garp 8G8), rinsed in PBST and incubated with anti-mouse IgG secondary antibodies conjugated to Alexa fluor 594 (Invitrogen) and the DNA dye TOTO-3 (Invitrogen). Images were collected using a 100 X objective (N.A. 1.45). Mouse sperm was immobilized on Cell-Tak (BD

Biosciences) coated MatTek plates (MatTek Corp), stained with antibodies against ankyrin-G and acetylated tubulin, and images were collected using a 63X objective, N.A. 1.4.

Immunoprecipitation and immunoblotting: Bovine rod outer segments were isolated from fresh bovine eyes by sucrose gradient centrifugation as described (S*13*). Our preliminary studies showed that ankyrin-G is very poorly solubilized by 1% Triton X-100. For this reason we adopted a strategy where we solubilized ankyrin-G present in ROS with sodium dodecyl sulfate (SDS) after performing chemical crosslinking to preserve protein interactions. To perform crosslinking, the ROS pellet was resuspended in phosphate buffered saline (PBS) containing 20% sucrose w/v, 1 mM NaEDTA , 0.1% Triton X-100, 2 mM AEBSF, 10 μM bestatin, 10 μM E-64, 10 μM leupeptin, 10 μM pepstatin (all protease inhibitors from EMD Biosciences) and 10 mM DTSSP cleavable crosslinking reagent (Pierce). Crosslinking was carried out on ice for 30 min and the reaction was quenched by 150 mM Tris-Cl. SDS was then added to a final concentration of 0.1 % to completely solubilize ankyrin-G and other protein complexes present in the mixture. Triton X-100 was added to the SDS lysates to a final concentration of 1% and the samples were centrifuged at 60,000 x g. The supernatant was subjected to immunoprecipitation using 10 μg of ankyrin-G, ankyrin-B or non-specific rabbit immunoglobulin and immunoprecipitates were collected on protein-G Dynabeads (Dynal). CNG-β1 was immunoprecipitated with 20 μg of the monoclonal antibody Garp 8G8 coupled to sepharose beads.

For immunoprecipitation from cells, HEK 293 cells (seeded at $2x10^7$ cells/10 cm plate) were co-transfected with 400 ng of ankyrin-G-GFP plasmid and 2.6 μg of plasmids

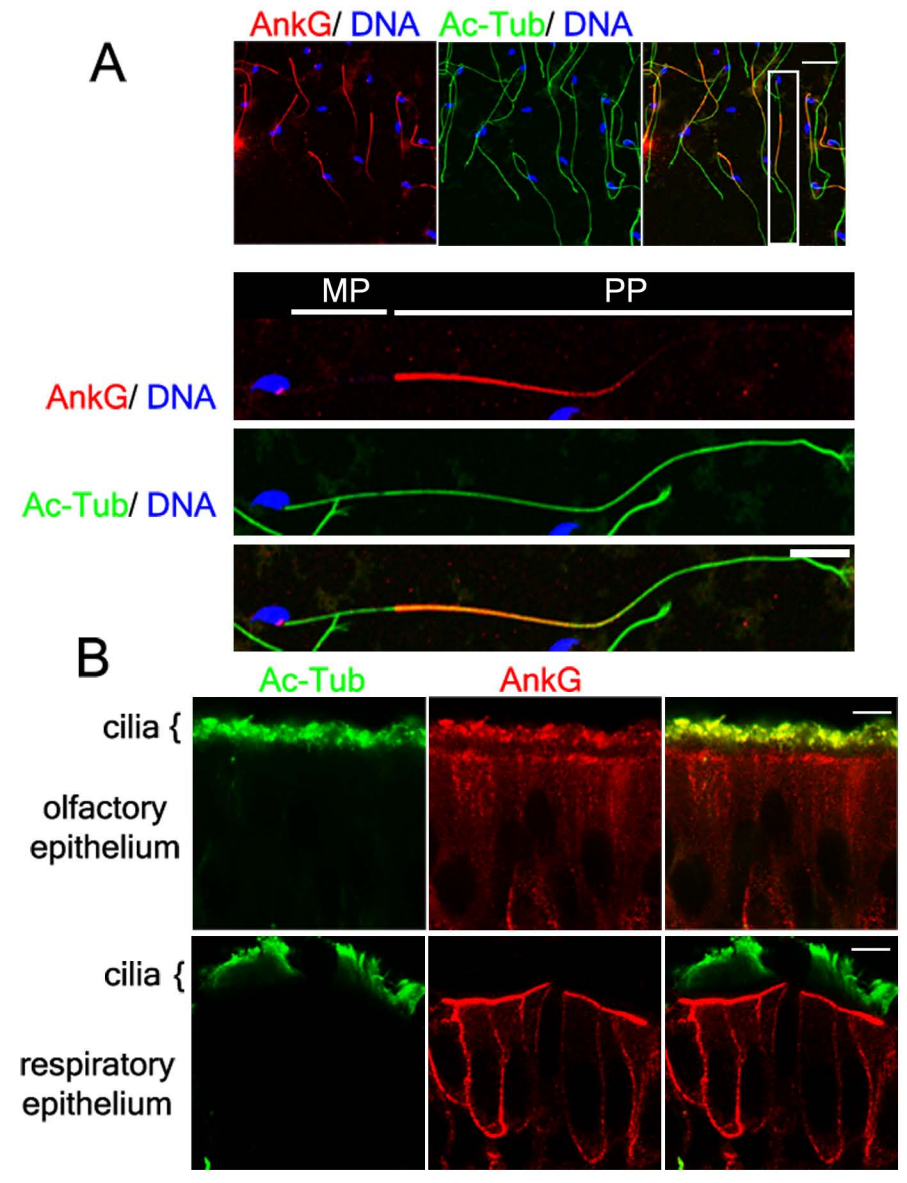
encoding either HA-neurofascin or HA-neurofascin-CNG-β1 fusion proteins using 120 μg of Effectene (Qiagen). For co-expression of channel subunits, cells were transfected with 3 μg of CNG-α1 and 1 μg of CNG-β1. Cells were lysed 48 h later using 1% Triton X-100 in PBS containing 20% sucrose w/v, 1 mM EDTA, 2 mM AEBSF, 10 μM bestatin, 10 μM E-64, 10 μM leupeptin, and 10 μM pepstatin (EMD Biosciences). The cell lysate was cleared of insoluble material by centrifugation at 100,000 x g. HA-neurofascin and HA-neurofascin-CNG-β1 fusion proteins were immunoprecipitated using a polyclonal anti-HA antibody. CNG-β1 was immunoprecipitated as described above. The supernatants and pellets from each immunoprecipitation of ROS lysates were resolved by SDS-PAGE and then immunoblotted with antibodies against ankyrin-G, CNG-β1 (Garp 8G8) and rhodopsin (1D4). Neurofascin fusion proteins and ankyrin-G-GFP from transfected HEK 293 cells were detected in immunoblots using anti-HA and anti-GFP antibodies, respectively. CNG-α1 and CNG-β1 were immunoblotted with the corresponding Pmc1D1 and Garp 8G8 antibodies. The antibodies were detected using 125I-labeled protein A/G (Pierce).

HEK 293 based membrane recruitment assay: The assay was performed as described in a previous study (S*2*). Briefly, 20 ng of plasmid encoding ankyrin-G-GFP and 180 ng of HA-neurofascin, HA-neurofascin CNG fusion protein or CNG-α1 were co-transfected using Effectene (5 μg, Qiagen) into HEK 293 cells grown on Matek Plates (3x104 cell/ 1.4 mm^2). For probing CNG-β1 interaction with ankyrin-G, a mixture of plasmids encoding CNG-β1 (50 ng), CNG-α1 (130 ng) and ankyrin-GFP (20 ng) was transfected into HEK 293 cells. Immunofluorescence was performed as described above.

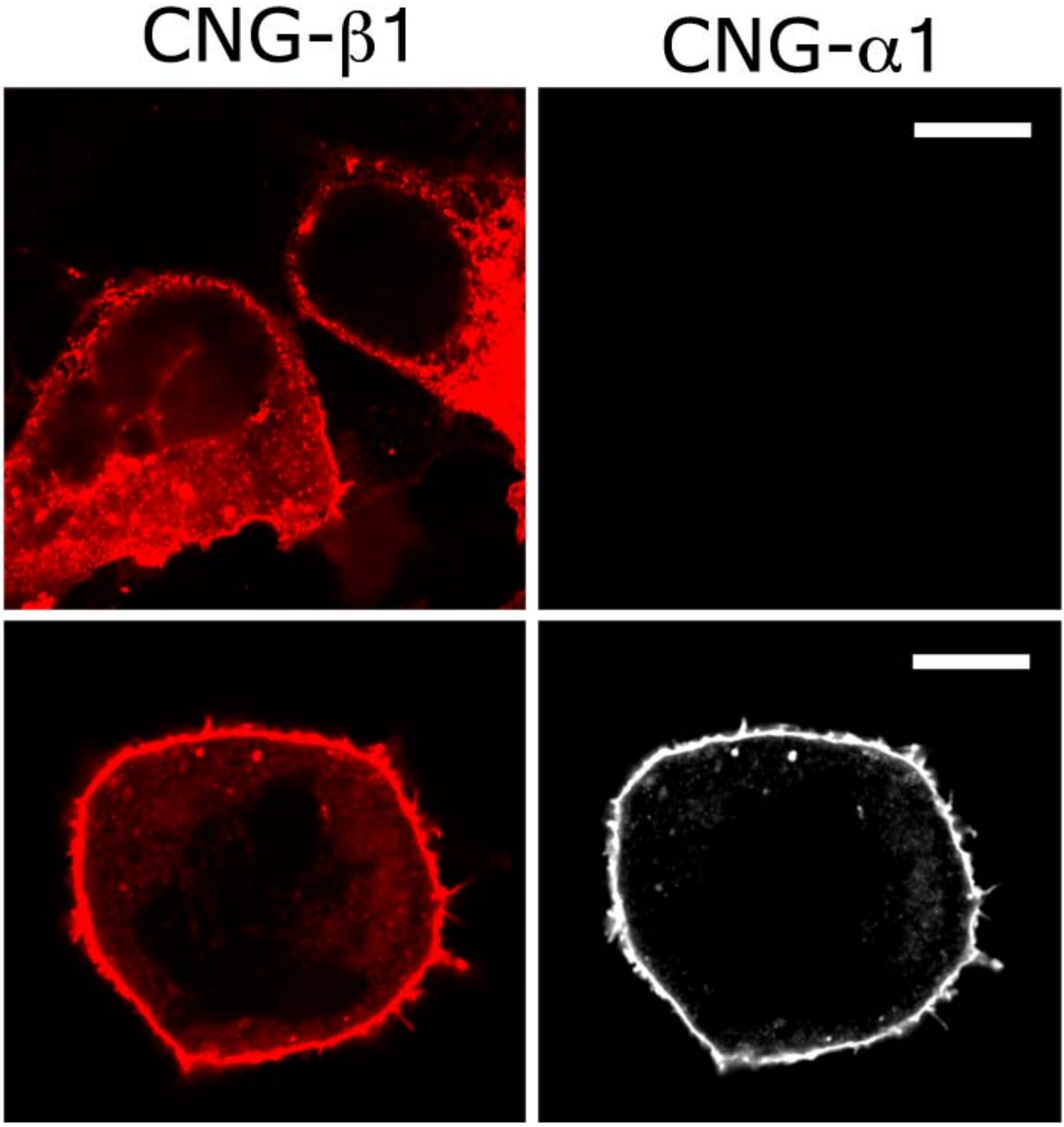
Generation of Transgenic Tadpoles

Transgenic *Xenopus laevis* tadpoles were produced using the restriction enzyme-mediated integration method described before (S*14, S15*) with modifications described in (S*16, S17*). Transgenic tadpoles, identified by expression of the GFP reporter in the lens, were collected at development stages 43-54 and immunodetection of proteins expressed in their rods was performed as described above. A minimum of four individual transgenic animals were evaluated for every DNA construct.

Kizhatil fig. S1



Kizhatil fig. S2



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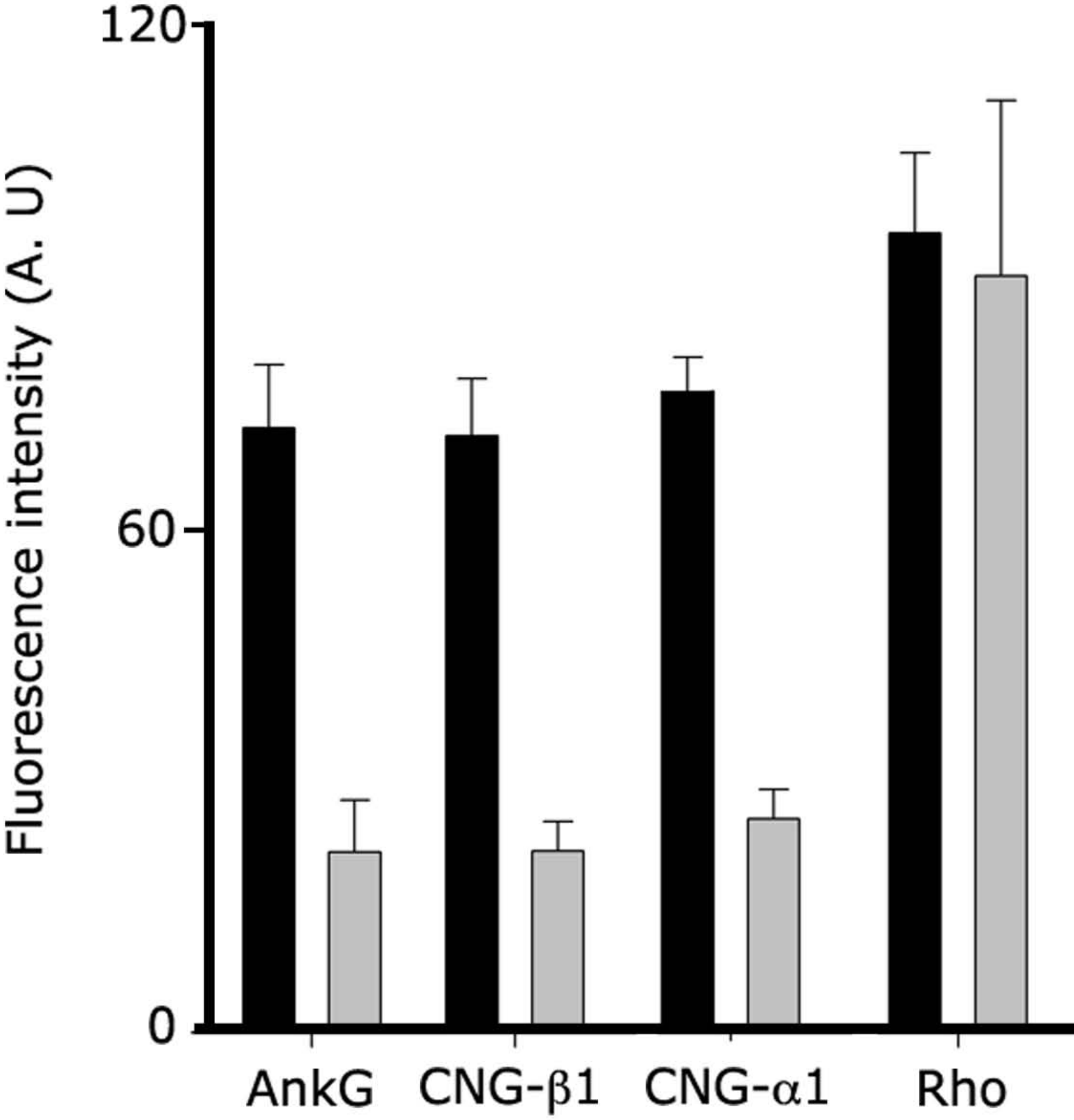

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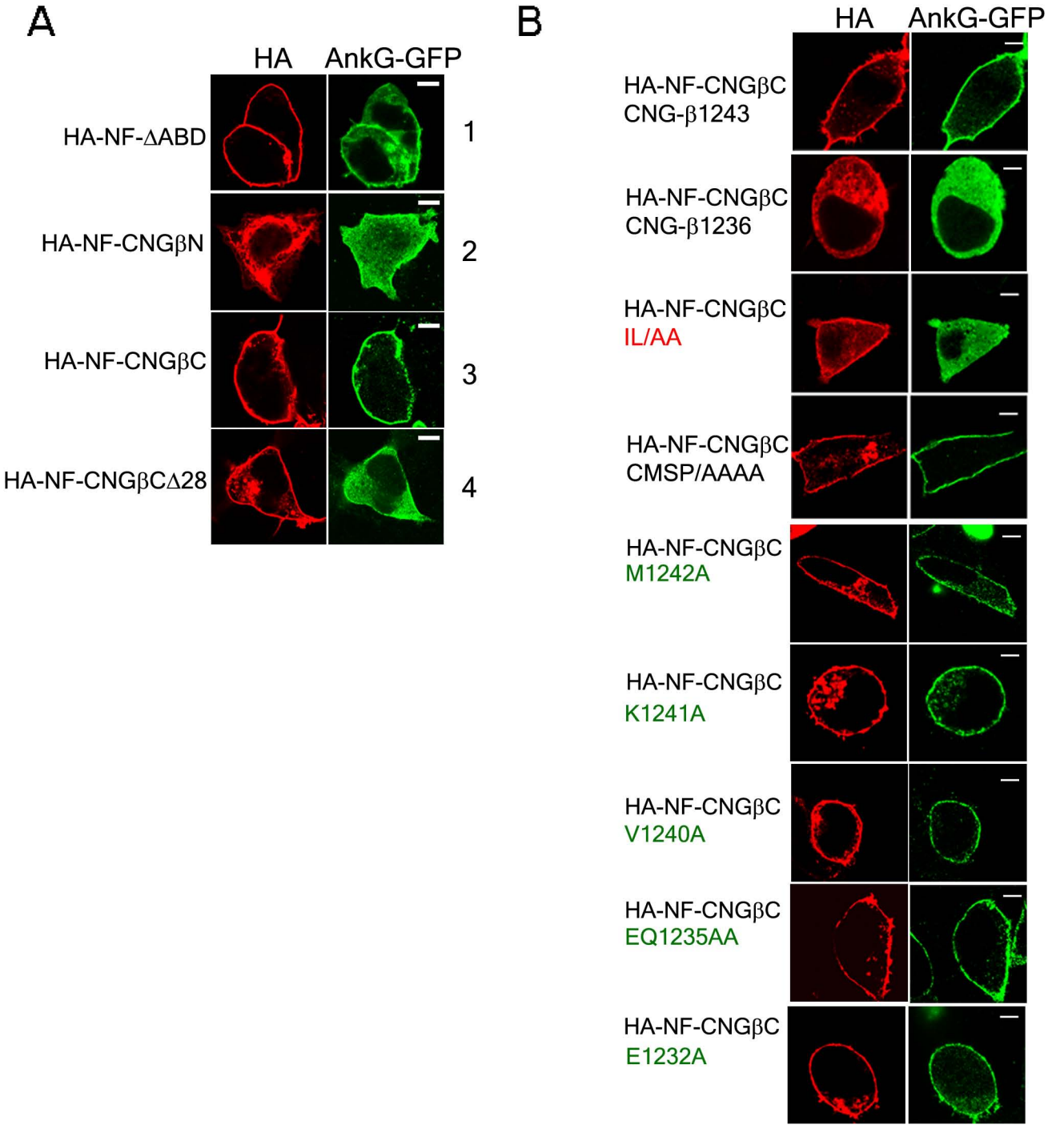
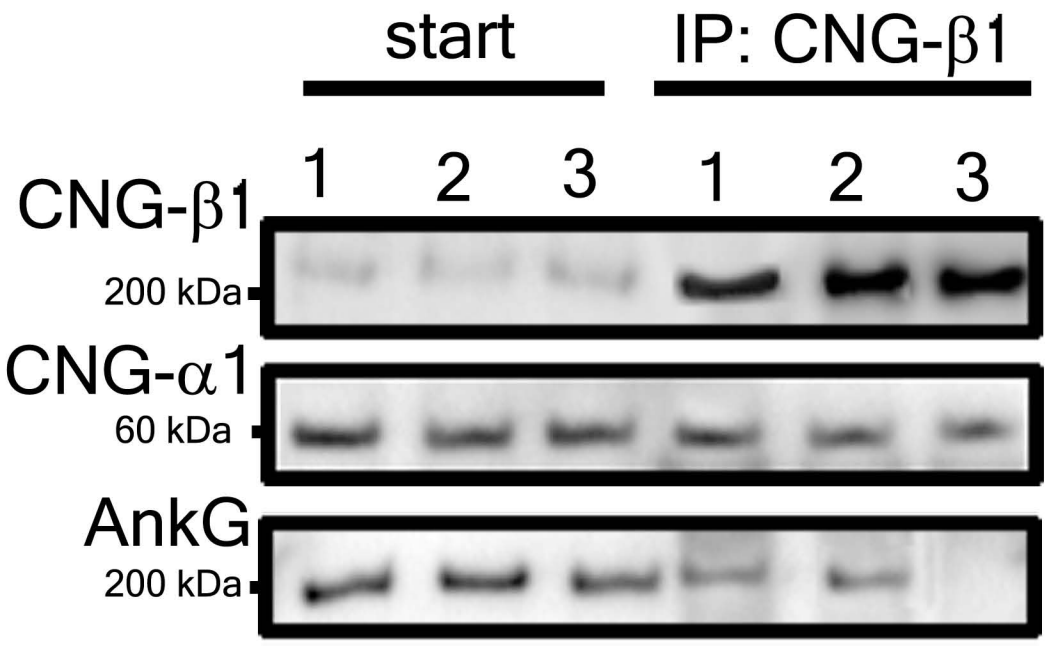
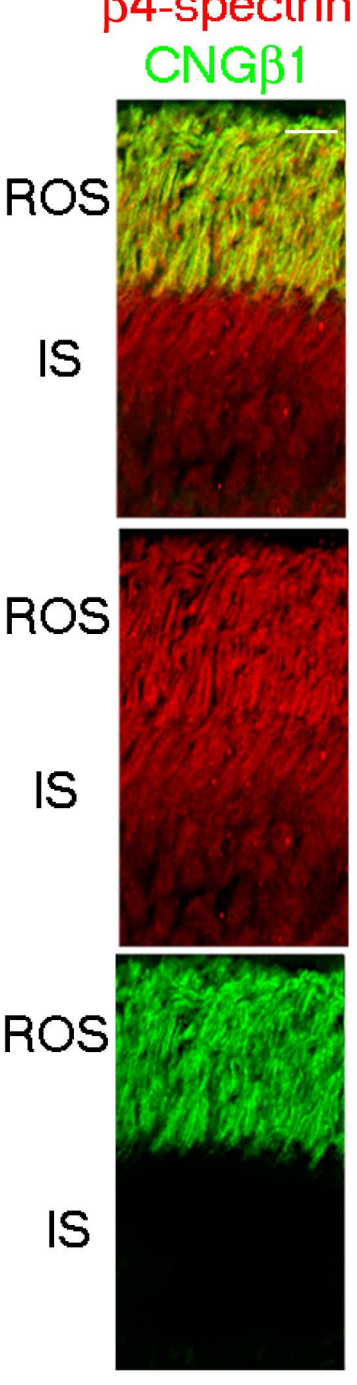


Kizhatil fig. S5



1. CNG-α1 + CNG-β1
2. CNG-α1 + CNG-β1- DAG
3. CNG-α1+ CNG-β1- DAG IIF/AAA

Kizhatil fig. S6



SUPPLEMENTARY FIGURE LEGENDS

Fig S1. Ankyrin-G localizes to the principle piece of the sperm flagellum and to olfactory cilia. (A) Ankyrin-G (AnkG, red) localizes to the proximal part of the principle piece (PP) of sperm flagellum, the same region where CNG-β1 has previously been shown to localize (S*18*). The sperm flagellum is marked by acetylated tubulin (Ac-tub, green). The DNA in the head of sperm is labeled in blue. Middle piece is abbreviated as MP. Upper panels show multiple sperm and the lower panel is an enlargement of the single sperm indicated in the box in the upper panel. Scale bars are 20 μm in the upper panels and 10 μm in the lower panels. (B) Ankyrin-G localizes to the olfactory cilia where CNG-β1 has previously been shown to localize (S*19*), but not to the respiratory cilia. Confocal section of mouse olfactory epithelium (upper panels) and respiratory epithelium (lower panels) in the nasal septum of a three day old mouse was immunostained with antibodies against acetylated tubulin (Ac-tub, green) to label cilia and against ankyrin-G (AnkG, red). Merged panels are shown to the right. Ankyrin-G co-stains the cilia of the olfactory epithelium but not that of the respiratory epithelium. Scale bars are 5 μm.

Fig S2. Expression of the beta subunit of CNG channel at the plasma membrane requires the co-expression of the CNG alpha subunit. The upper panels show the intracellular localization of CNG-β1 (red) subunit when expressed alone in HEK 293 cells. Lower panels show the plasma membrane localization of CNG-β1 (red) when coexpressed with CNG-α1 in HEK 293 cells (white). Scale bars are 10 μm.

Fig S3. Effect of ankyrin-G depletion in rods on the levels of ROS resident proteins as measured by fluorescence intensity**.** Fluorescence intensity of ankyrin-G (AnkG), CNG-

β1, CNG-α1 and rhodopsin (Rho) in either pFIV control transfected (black bars) rod photoreceptor outer segments or pFIV ankyrin-G shRNA transfected (grey bars) rod photoreceptor outer segments. Transfected cells were identified by expression of GFP from a pCAAGS-GFP plasmid mixed in with the control or shRNA plasmids at a 10-fold lower concentration. Data are mean +/- SD (n= 50 GFP positive ROS). An important caveat regarding interpretation of fluorescence intensity of rhodopsin is as follows. Rhodopsin in the outer segment represents a very specific case in which the fluorescence intensity may not accurately reflect its concentration. This is because rhodopsin concentration in the outer segment is ~3 mM which corresponds to ~100,000 molecules in each of 800 discs. For this reason its immunostaining signal can be saturated and therefore changes in rhodopsin density may not be accurately reflected by immunostaining intensity.

Fig S4. Mapping of the ankyrin-G binding site within the 28 C-terminal amino acids of CNG-β1. The data in all panels represent results from the HEK 293 plasma membrane recruitment assays using AnkG-GFP and HA-neurofascin/CNG-β1 fusion proteins. cDNAs transfected into HEK 293 cells in addition to AnkG-GFP are listed on the left of each panel. The HA-neurofascin/CNG-β1 fusion proteins were detected using the anti-HA antibody and ankyrin-G-GFP was detected using the antibody against GFP. Scale bars are 10 μm. (A) The ankyrin-G binding site maps to the 28 C-terminal amino acids of CNG-β1. The results are summarized in Fig. 3A with the numbers on the right side of each panel corresponding to the schematic diagrams in Fig. 3A. (B) Identification of the IL1237 residues of CNG-β1 as the amino acids critical for ankyrin-G binding. The two upper panels illustrate the hCNG-β1243 and hCNG-β1236 deletion mutations shown in

Fig. 3C. Other panels illustrate the results of the alanine-scanning mutagenesis of individual amino acid residues within the CNG-β1 C-terminus. The ankyrin-G binding residues IL1237 are shown in red.

Fig S5. CNG-β1/dystroglycan chimera co-immunoprecipitates CNG-α1 subunit and endogenous ankyrin-G in HEK 293 cells. cDNA encoding one of three CNG-β1 constructs was co-transfected into HEK 293 cells along with the cDNA encoding the CNG-α1 subunit. The first construct was human CNG-β1, used as a control. The second encoded a CNG-β1/dystroglycan (DAG) chimera in which the ankyrin-G binding site of CNG-β1 was substituted with 14-amino acids containing the ankyrin-G binding site of beta-dystroglycan. The third construct encoded a CNGβ1/DAG chimera bearing the IIF/AAA mutation in the ankyrin binding site. Immunoprecipitation was performed using an N-terminal specific CNG-β1 antibody. Starting material is shown on the left and the immunoprecipitates on the right.

Fig S6. Localization of β-4-spectrin in rod photoreceptors. Beta-4-spectrin (red) was labeled using a rabbit antibody (gift of Michele Solimena, Medical Faculty Carl Gustav Carus of the University of Technology, Dresden, Germany (S*20*)) and is expressed in both inner segments (IS) and ROS of mouse rod photoreceptors. ROS were labeled using an antibody against CNG β1 (green). The scale bar is 5 μm.

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